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Paromomycin: uptake and resistance in *Leishmania donovani*

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Abstract

Paromomycin is currently in phase IV clinical trials against leishmaniasis. In the present work we elucidate the effect and mechanism of uptake of paromomycin in *Leishmania donovani*. The *in vitro* sensitivities of both promastigotes and amastigotes were determined to this aminoglycoside. Association of paromomycin with *Leishmania donovani* involved a rapid initial phase that was non-saturable up to 1 mM of the drug. This initial phase was largely independent of temperature and not affected by metabolic inhibitors. Poly-lysine, a membrane impermeant polycation, caused profound inhibition of this association of the drug with the parasite indicating that it represented a binding of the cationic paromomycin to the negatively charged leishmanial glycocalyx. After 72 hours of exposure to the drug the mitochondrial membrane potential was significantly decreased, indicating that this organelle might be the ultimate target of the drug. Both cytoplasmic and mitochondrial protein synthesis were inhibited following paromomycin exposure. A line selected for resistance to the drug showed reduced paromomycin accumulation associated with a significant reduction in the initial binding to the cell surface. The drug induced reduction in membrane potential and inhibition of protein synthesis were less pronounced in the resistant strain in comparison to the wild-type.

Keywords

L. donovani; paromomycin; transport; resistance; protein synthesis; mitochondria

1. Introduction

Leishmania donovani, a flagellated protozoan parasite, is the causative agent of visceral leishmaniasis (VL). The current increase, to epidemic proportions, in leishmaniasis throughout the world and the emergence of visceral leishmaniasis as an important opportunistic infection among people with human immunodeficiency virus-1 (HIV-1) infection [1,2] has increased the need for new treatments for this intracellular infection. The alarming emergence of resistance to currently used drugs exacerbates this need for new drugs.

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Pentavalent antimonials are the standard first-line treatment for leishmaniasis [3,4], although resistance is a growing problem [5]. A parenteral formulation of aminosidine (paromomycin) has recently been approved for leishmaniasis treatment in India [6–8], where it is in phase IV trials. Paromomycin (PR) is an aminoglycoside antibiotic that has been used for various clinical infections. The drug is used against gram-positive and gram-negative bacteria [9] and parasitic infections, including giardiasis, amoebiasis [10] and cryptosporidiosis [11]. Paromomycin has been used for the treatment of both VL, in a parenteral formulation, and cutaneous leishmaniasis (CL) in both topical and parenteral formulations [12,13].

The mechanism of antibacterial effect of paromomycin has been well documented, with the drug acting to inhibit protein synthesis through its interaction with ribosomal RNA subunits [9]. Moreover, at least three mechanisms of aminoglycoside resistance are recognized in prokaryotes: reduced uptake or decreased cell permeability, alterations at the ribosomal binding sites, or production of aminoglycoside modifying enzymes [14].

Previous studies have suggested the involvement of mitochondrial membrane potential, ribosomes and respiratory dysfunction in the mode of action of paromomycin in *Leishmania* spp. [15–17]. In addition, a paromomycin-resistant *Leishmania donovani* line, developed by stepwise increase in drug pressure, showed decreased paromomycin uptake compared with a wild-type parental line [18].

In this paper we describe the sensitivity of both promastigote and amastigote forms of Indian *Leishmania donovani* to paromomycin *in vitro*. We also report on paromomycin uptake, its effect on protein synthesis and mitochondrial membrane potential in wild-type *Leishmania* and a laboratory derived resistant line.

2. Materials and methods

2.1. Materials

[³H]- Paromomycin [0.6 Ci/mmol] was custom synthesized by Moravek Biochemicals Inc. All other chemicals were from Sigma (St. Louis, MO) except AlamarBlue™ which was supplied by AbD Serotech Ltd., Oxford, UK. Sodium antimony gluconate (SAG) was from Glaxo-Wellcome. [¹⁴C]-Isoleucine (300 Ci/mmol) was obtained from BRIT, Mumbai (India).

2.2. Strains and culture conditions

Promastigotes of *Leishmania donovani* strain AG83 (MHOM/IN/80/AG83) wild-type (WT) were routinely cultured at 22°C in modified M-199 medium supplemented with 0.13 mg/ml penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Life Technologies Scotland, UK). The WT strain of *L. donovani* was cloned on semi solid M-199 containing 1% Bacto Agar (Difco), 10% heat inactivated fetal bovine serum and 0.13 mg/ml penicillin and streptomycin. Colonies were picked and transferred separately into liquid M-199 [19]. Axenic amastigotes were obtained after transformation of promastigotes to amastigotes as described by Debrabant et al. [20].

2.3. Development of a paromomycin resistant strain

The cloned wild-type cells were cultured in M-199 medium (with supplements), in the presence of 20 µM of paromomycin. The cultures were stabilized for six sub-cultures before increasing the drug level at one passage per week. The line growing in 20 µM paromomycin was plated on M199-agar plates in the presence of the drug and a single colony was picked for culture. The cultures were subjected to step-wise increasing drug pressure with final

concentration of 50 μ M. Cells from cultures were again cloned on semi-solid medium in the presence of drug. The clone was designated as PRr that was resistant to 50 μ M of paromomycin. It revealed an IC_{50} (50% inhibitory concentration) of 145 μ M (making it three-fold less sensitive to paromomycin than wild-type). Our attempts to further increase the drug pressure were not successful even over a three month time period. Stability of resistance was checked at two weeks, four weeks and eight weeks after removal from drug pressure.

2.4. Cross-resistance studies

Cross resistance to other drugs was determined by measuring the 50% inhibitory concentration (IC_{50}) with the colorimetric AlamarBlue™ dye reduction assay [21]. To measure the IC_{50} values for different drugs, the parasites were seeded into 96-well plates at a density of 5×10^5 cells/ml in 200 μ l medium containing different drug concentrations. Alamar blue (20 μ l) was added and after 72 h of incubation at 24°C, the fluorescence was monitored at 545 nm excitation wavelength and 590 nm emission wavelength. Growth inhibition was calculated with respect to the fluorescence intensity measured with untreated control cells.

2.5. Effect of drug on an amastigote-macrophage model

Stationary phase *Leishmania* promastigotes expressing the luciferase gene, pGL- α NEO α LUC were used to infect macrophages. J774A.1 macrophages were maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a CO₂ incubator (5% CO₂). Sub culturing was carried out at 72 h when the cells reached confluence [19]. Briefly, J774A.1 murine macrophages (1×10^5 cells/ petridish) were infected with 1×10^6 promastigotes (expressing the luciferase gene, pGL- α NEO α LUC, kindly given by Dr. Marc Ouellette, Canada) in M199 media with 10% FBS [22]. After 3 h, the non-internalized parasites were washed off and paromomycin was added at different concentrations. After 3 days of drug exposure, plates containing adherent macrophages were washed and luciferase activity was determined as reported earlier [19,22]. The 50% inhibitory concentration (IC_{50}) was determined from a graph representing different concentrations of drug plotted against Relative Light Units (RLU) produced by luciferase expressing parasites.

2.6. Uptake studies in *Leishmania donovani*

Parasites were harvested during the mid-logarithmic phase of growth by centrifugation at $2,100 \times g$, for 10 min at 4°C. Cells were then washed twice with phosphate buffered saline (PBS) supplemented with 1% D-glucose (PBSG) at pH 7.4. Cells were resuspended in this buffer at densities indicated in the text and figure legends. Parasite suspensions (100 μ l, containing 2×10^7 cells) were warmed to 25°C and mixed with 100 μ l of assay buffer containing labeled molecule plus or minus other test compounds at the concentration indicated. Transport was terminated after times indicated in the figure legends by the rapid separation of parasites from the buffer components by centrifugation through a 9:1 mixture of dibutylphthalate (specific gravity, 1.04) and mineral oil (specific gravity, 0.875 to 0.885). The sample tubes were immediately flash frozen in liquid nitrogen, and the tubes were cut to separate the pellet from the transport medium. The pellet was dissolved in 2% sodium dodecyl sulfate (200 μ l) and 3 ml of scintillation fluid (Cocktail T, Spectrochem. Pvt. Ltd., India). These were left overnight to remove the effects of chemiluminescence, and then incorporated radioactivity was counted in a scintillation counter (Beckman Coulter)

2.7. Inhibition studies

The effects of various metabolic inhibitors, endocytotic inhibitors, ionophores and uncouplers on paromomycin uptake in wild-type promastigotes were studied. The inhibition

studies were performed by resuspending cells in PBSG containing inhibitors at concentrations and times as indicated in the figure legends at 25°C. After this treatment, 100 μM [^3H] paromomycin was added to the mixture and uptake was measured for 10 min. Accumulated label was counted as described above. In order to determine the reversibility of paromomycin association, cells were loaded with 100 μM paromomycin for 10 min at room temperature. After exposure to the drug, an aliquot was immediately centrifuged through oil and frozen in liquid nitrogen. Retained paromomycin was determined as described above. The remaining cells were centrifuged, washed three times with PBSG, resuspended in drug-free PBSG and incubated at 25°C or 4°C. Aliquots of cells were removed, and intracellular paromomycin was determined over a range of time points.

2.8. Effect of drug on total protein synthesis of promastigotes

Log phase *Leishmania donovani* promastigotes were seeded in 200 μl of M-199 medium with 10% FCS in 96 well plates at a density of 1×10^6 cells/ml. Cells were treated with varying concentrations of paromomycin for 24 h and incubated at 22°C. Assays were carried out in duplicate using untreated cells as controls. [^{14}C]-isoleucine (2 $\mu\text{Ci/ml}$) was added to the cultures for 10 min at the end of treatment. The cells were lysed and contents precipitated with trichloroacetic acid (TCA) (final 5%, w/v) and the plates were kept at 4°C for 20 min. The TCA precipitated macromolecules were transferred to the multi-well filter plates (AcroPrep™ filter plates, Pall Corporation), and washed 3 times with 5% TCA. The plates were then dried at 37°C. Scintillation fluid was then added to each well and readings were taken in the liquid scintillation counter (1450 Microbeta Wallac). In order to study the effect of paromomycin on cytoplasmic and mitochondrial protein synthesis the log phase *Leishmania donovani* promastigotes were seeded as described above. Assays were carried out in duplicate using untreated cells as controls. Cells were treated with 1 mM paromomycin [17] for 3 h followed by addition of either 1 mM chloramphenicol or 70 μM cycloheximide. In parallel, cells were treated with the same concentration of either chloramphenicol or cycloheximide alone. Labeled isoleucine (2 $\mu\text{Ci/ml}$) was added and the incubation continued for another 10 min. The incorporation of the isoleucine was determined as described above.

2.9. Determination of mitochondrial transmembrane potential

Wild-type, paromomycin-treated cells and resistant cells were stained with 0.05 μM rhodamine 123 for 30 min to determine the relative mitochondrial membrane potential. Mitochondrial membrane potential was assessed by flow cytometry as previously described [23]. Data were gathered with a FACS Calibur (Becton Dickinson, Oxford, UK) flow cytometer equipped with an argon-ion laser (15 mW) tuned to 488 nm. Rhodamine fluorescence was collected in the photomultiplier tube designated FL-1, which is equipped with a 530/30 nm band pass filter. Data analysis was carried out with CellQuest (Becton Dickinson, Oxford, United Kingdom) software.

2.10. Data analysis

Data were statistically analyzed by student's t-test. A p value of < 0.05 was considered to be significant. All experiments were repeated twice or thrice with triplicates in each set. Data was fitted to the appropriate equations using the GraphPad Prism® Version 4.0 software package.

3. Results

3.1. Sensitivity of *L. donovani* to paromomycin

Paromomycin inhibited the growth of *L. donovani* promastigotes in a dose dependent manner; ($IC_{50} = 50 \pm 2.5 \mu M$). Intracellular macrophage-amastigotes were somewhat more sensitive ($IC_{50} = 8 \pm 3.2 \mu M$).

3.2. Paromomycin association with *Leishmania*

To determine uptake of paromomycin in *L. donovani* promastigotes cells were exposed to extra cellular paromomycin (100 μM) and uptake was permitted to proceed for 4 h at 25° C (Fig. 1A). By 4 h, drug had accumulated to ~80 pmol/10⁷ cells. The rate of uptake increased after 16 hours (data not shown). In axenic amastigotes by 4 h at 25° C drug had accumulated to ~91 pmol/10⁷ cells (Fig. 1B).

To determine whether association of drug with promastigotes was saturable, parasite-associated paromomycin was measured with a range of substrate concentrations (15.6 μM to 1.0 mM) for 10 min. The results showed that the rate of association increased in a near linear fashion with paromomycin concentration (Fig. 1C). A similar pattern of continuous increase of drug associated with amastigotes was obtained over a concentration range from 0.25 mM – 1.0 mM (data not shown).

Association of paromomycin with *L. donovani* as a function of temperature was also studied. Parasites were incubated with 100 μM paromomycin at 25°C and 4°C. Associated drug was measured after 10 min. The decrease in associated drug at 4°C was only around 25% of that seen at 25°C (Table 1).

This relative lack of temperature sensitivity and lack of saturation is reminiscent of the situation in bacteria where aminoglycoside antibiotics first bind to negatively charged components of the cell membrane [24]. Poly-(L)-lysine, a membrane impermeant polycation, has been used previously to block cation binding sites on cell surfaces [25]. We also used this reagent to test the effect on uptake of paromomycin in *Leishmania*. 0.01% poly-(L)-lysine reduced parasite-associated paromomycin by some 66% implying an absorptive role of the negatively charged membrane. Poly-alanine (0.01%), used as a control, had no effect (Fig. 2).

The binding of aminoglycosides to bacterial membranes is a reversible process. We tested whether *Leishmania* pre-exposed to paromomycin also went onto release this compound (Fig. 3B). Paromomycin was rapidly lost from the cells indicating that binding was reversible with most of the drug associated with cells at 1 h is probably bound to the extracellular surface with only a small fraction found inside the cells.

3.3. Effect of endocytotic inhibitors on paromomycin uptake

To further explore the possibility that paromomycin enters leishmania by endocytosis after first binding to the cell membrane, we looked at the effect of inhibitors of endocytosis [26–28] on its intracellular accumulation. Treatment for 1 h with the microtubule-disrupting reagent, vinblastin (10 μM), prior to the addition of paromomycin, resulted in reduced net association (to $66 \pm 12\%$ of the control level) as did treatment with cytochalasin D (10 μM) (to $71 \pm 13.0\%$ of the control level). When cells were treated with N-ethylmaleimide (NEM) (10 μM), an endocytotic inhibitor reacting with the –SH functions, paromomycin uptake was found to decrease to $58 \pm 9\%$ of the control. Nystatin (1h; 25 $\mu g/ml$), an agent that complexes cholesterol and alters the structure and function of glycolipid microdomains and

caveola [29] showed no effect, suggesting, that the uptake of paromomycin occurred by the caveola-independent pathway.

The weak bases NH_4Cl (either at 10 mM (5 min) or 30 mM (1 h)) and chloroquine (100 μM ; 30 min and 1h), which de-acidify lysosomal compartments [30], had no effect, indicating that the drug continues to enter through endocytosis in the presence of these lysosomal function inhibitors over a one hour time course.

3.4. Effect of metabolic inhibitors on paromomycin uptake

The effects of various inhibitors of metabolism and ionophores were also tested. The cellular ATP pool was diminished by pre-incubating the cells in 0.1% sodium azide and 50 mM 2-deoxy-D-glucose for 1 h at 25° C and paromomycin uptake was measured after 10 min. This treatment resulted in a decrease in the extent of internalization of the drug to $66 \pm 8\%$ of the control level (Table 1) indicating a possible role for energy dependent processes in net accumulation over 1 h. However, in the absence of a control whose uptake might proceed at the same rate regardless of the availability of ATP to the cell, the conclusion remains tentative. Potassium cyanide, an inhibitor of the electron transport chain showed a marginal but significant inhibition of accumulation ($P < 0.05$) as did another inhibitor of mitochondrial metabolism, sodium azide ($P < 0.05$). Oligomycin, an inhibitor of mitochondrial H^+ -ATPase [31] and Na^+/K^+ -ATPase [32] had no effect and nor did valinomycin, a potassium ionophore [33] or the protonophore CCCP [34,35]. Ouabain, however, which interferes with the transmembrane sodium ion gradient (inhibitor of the plasma membrane Na^+/K^+ -ATPase) [36] had a significant impact on the net accumulation of paromomycin ($P < 0.05$).

3.5. Paromomycin-resistant (PRr) *L. donovani* promastigotes

Paromomycin resistant *L. donovani* were selected by serial passage in increasing concentrations of the drug. The cloned line, designated PRr, displayed ~3-fold resistance to paromomycin with no cross-resistance to the other anti-leishmanial drugs, namely, pentavalent antimony, pentamidine, amphotericin B and miltefosine (Table 2). Another aminoglycoside, neomycin, a structural analog of paromomycin, at concentrations as high as 600 μM resulted in only 30% inhibition of the parasite growth both in the wild and the PRr strain. Earlier studies have also reported weak effect of neomycin on *L. donovani* growth [17]. The PRr strain, grown for up to 2 months in drug free medium, showed no loss of resistance (data not shown).

Uptake of 100 μM paromomycin was measured over 4 h at 25°C in the paromomycin-resistant line. Significantly less drug associated with the resistant line in comparison to the sensitive strain (30 pmol/ 10^7 cells at 4 hr, around half of the quantity found in wild-type cells) (Fig. 3A). The rate at which bound drug was lost from resistant lines when washed and replaced in drug free buffer was, however, similar to that seen for wild-type (Fig. 3B).

3.6. Effects of paromomycin on cellular function

Paromomycin affected the incorporation of the labeled protein precursors in wild type cells while resistant cells were not affected by up-to 100 μM of the paromomycin. At 1mM, paromomycin inhibited total protein synthesis by 21% and 2% in the PRr and wild-type strain respectively. Cycloheximide and chloramphenicol, inhibitors of cytoplasmic and mitochondrial protein synthesis respectively, both had an additive effect on paromomycin's inhibitory effect which could indicate that both cytoplasmic and mitochondrial protein synthesis are effected (Table 3).

We also tested the ability of the parasites to accumulate the hydrophobic cation, rhodamine 123 (Rh123), which is widely used as an indicator of mitochondrial membrane potential [23]. After 72 h of exposure to 150 μ M paromomycin, Rh123 fluorescence was significantly decreased indicating that mitochondrial membrane potential is affected by drug exposure. Interestingly the mitochondrial membrane potential also seems to be diminished in resistant parasites compared with wild type (Fig 4).

4. Discussion

The present work aimed to examine the mechanism of uptake of paromomycin (PR), its mode of action and also how resistance to it evolves in *Leishmania donovani*. This aminoglycoside was previously proposed exert its effect on RNA synthesis and to modify membrane fluidity and permeability [17]. Previous work indicated effects on mitochondrial activity inducing respiratory dysfunction with associated changes to mitochondrial membrane potential [16]. Resistance was shown to relate to reduced association of drug with the parasites [18]. Here we have confirmed these previous findings. In the present study we have carried out the first detailed study into uptake of paromomycin using a radiolabelled form of the drug. Furthermore we have extended studies to the amastigote form of the parasites.

The uptake experiments have shown that association of paromomycin with the parasites was not saturable up to 1 mM. Moreover, exposing *Leishmania* to drug at room temperature or on ice had minimal effect on the kinetics of this association, thereby indicating that a carrier-mediated process, which should be much reduced at 4°C, had little impact on the initial measured association of paromomycin with *Leishmania*. The fact that the membrane impermeant cation poly-(L)-lysine caused profound inhibition of this association of the drug with the parasite, suggesting it competed with binding of the cationic paromomycin to the negatively charged leishmanial glycocalyx. After returning parasites to paromomycin free medium, drug was rapidly lost from the membrane. Similar effects are known for bacteria where aminoglycosides associate with lipopolysaccharide (LPS) and other anionic components of the bacterial cell surface [24]. Promastigotes of *Leishmania* possess a highly negatively charged lipophosphoglycan structure (LPG) as a major component of their cell surface [37,38]. Several glycosylphosphatidylinositols have been reported to accumulate in the endosomes [33,39]. Whilst it is tempting to speculate that paromomycin associates with LPG at the Leishmanial surface the fact that similar uptake phenomena are seen in amastigote forms of the parasites, where LPG is greatly reduced in abundance, suggests other components must also be involved in binding. After binding to the surface, internalization appears to proceed by endocytosis since a number of typical inhibitors of endocytosis (vinblastin, cytochalasin D, NEM and placing cells on ice) all reduced the rate of uptake of the drug in the order of 35%. The fact that the net reduction in uptake was rather modest would be expected if most of the measured paromomycin is associated with the cell surface; the quantity endocytosed over the one hour period being relatively small. By subtracting the estimated cell-surface binding material the inhibition of uptake via the proposed endocytotic route would become much higher. Unfortunately, the toxicity of the inhibitors of endocytosis to *Leishmania* made it difficult to use longer uptake periods. Once inside the cell it is possible that the drug enters the mitochondrion, where the high membrane potential will serve to drive uptake of this cationic agent.

We also selected cells for 3 fold resistance to paromomycin. Cross resistance to other leishmanicides was not evident. A putative binding site on the small-subunit rRNA gene was identified. No changes in that site were evident in PCR products covering the site in the resistant line (data not shown) consistent with earlier reports in paromomycin resistant *L. tropica* [40]. Our comparative drug uptake studies show that the paromomycin resistance in

L. donovani is associated with reduced accumulation of drug in the resistant promastigotes with a significant reduction in the initial binding of drug to the cell surface. The reduced accumulation of drug in these parasites leads to protein synthesis being retained intact. Of interest is the fact that it proved extremely difficult to select for high level paromomycin resistance. Moreover, the fact that the paromomycin resistant cells retained sensitivity to other drugs is of significance in indicating that should resistance emerge in the field these parasites can probably be treated with existing agents.

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(A)

(B)

(C)

Fig. 1.

Paromomycin uptake by wild-type promastigotes as a function of treatment time and concentration. (A) Promastigotes in the exponential phase of growth were incubated with 100 μ M paromomycin at 25°C, and paromomycin uptake was measured in promastigotes between 10 min and 4 h paromomycin. (B) uptake in axenic amastigotes between 10 min and 4 h. (C) Promastigotes in the exponential phase of growth were incubated with varying concentration of paromomycin between 15.6 μ M and 1 mM at 25°C and paromomycin uptake was measured at 10 min. Each point represents the mean $\pm$ S.D. of at least triplicate observations.

Fig. 2.

Effect of membrane impermeant polycation on the uptake of paromomycin. Paromomycin uptake was measured after the pretreatment of the promastigotes in absence (control) or in presence of 0.01 %, poly-(L)-lysine or 0.01% poly-alanine for 15 min. Each point represents the mean $\pm$ S.D. of at least triplicate observations. (*) not statistically different from the control group. (**) is statistically different at $P < 0.005$ when compared to the values obtained for controls.

Fig. 3. Comparative study of paromomycin influx and efflux in the wild-type and resistant cells. (A) Paromomycin uptake by wild-type and resistant promastigotes as a function of treatment time. Promastigotes in the exponential phase of growth were incubated with 100 μ M paromomycin at 25 °C and uptake was measured between 10 min and 4 h. Each point represents the mean $\pm$ S.D. of at least triplicate observations. (■) wild-type promastigotes; (▲) paromomycin-resistant promastigotes. (B) Efflux of paromomycin by promastigotes. Wild-type and resistant parasites were incubated with 100 μ M paromomycin at 25°C for 10 min, centrifuged, washed two times with PBSG, and resuspended in drug-free PBSG. Retained paromomycin was measured between 15 sec and 1 h. Each point represents the

mean $\pm$ S.D. of at least triplicate observations. (■) wild-type promastigotes (▲) paromomycin-resistant promastigotes

Fig. 4.

Flow cytometry analysis of wild-type, paromomycin treated *L. donovani* promastigotes, paromomycin-resistant strain (PRr) and PRr treated with paromomycin. The figure shows a set of histograms, representative of three separate experiments. Promastigotes of the wild-type and resistant type, treated or untreated with paromomycin were incubated with rhodamine 123 as described in the materials and methods. Cell suspensions were subjected to flow cytometry and the fluorescence distribution was plotted as frequency histograms. Figure shows a set of histograms, for the populations: (a) unstained population, (b) wild type untreated, (c) wild type treated with 150 μ M of paromomycin for 72 h, (d) paromomycin-resistant strain and (e) paromomycin-resistant strain treated with 150 μ M of paromomycin.

Table 1

Effect of mitochondrial metabolism inhibitors on the uptake of paromomycin

Treatment	Inhibitor concentration	Mean % of control accumulation
Control-25°C	—	100.0
4°C	----	75.5 ± 5.5
Cyanide	1 mM	71.9 ± 10 [*]
Azide	5 mM	81.4 ± 1.5 [*]
Oligomycin	15 µM	115.9 ± 20
Valinomycin	10 µM	98.9 ± 6.4
CCCP	10 µM	88.1 ± 8.9
Ouabain	1mM	69.6 ± 8.9 [*]
Azide + 2-deoxy-D-glucose	0.1% + 50 mM	66.8 ± 8.0 [*]

Cells were pretreated with inhibitors at 25 °C for 10 min and then incubated with 100 µM paromomycin for 10 min in PBSG. All values are given as a percentage of the wild-type control. Results are means ± S.D. of at least two independent experiments.

* is statistically different at $P < 0.05$, when compared to the values obtained for control 25°C.

Table 2Cross-resistance profile of wild-type (WT) and paromomycin-resistant (PRr) *L. donovani* promastigotes

Drug	Mean IC ₅₀ ± S.D. (μM)	
	Wild-type	PRr
Paromomycin	50.0 ± 2.5	145 ± 3 ^{**}
Pentamidine	1.2 ± 0.3	1.8 ± 0.4 [*]
Amphotericin B	0.05 ± 0.01	0.07 ± 0.01 [*]
Sb V	35 ± 4.2	23 ± 1.4 [*]
Miltefosine	15.6 ± 0.25	10.4 ± 0.4 [*]

IC₅₀s were determined in promastigotes of paromomycin-sensitive and Paromomycin-resistant strains after 72 h of drug addition. IC₅₀ are given as means ± S.D. of at least two independent determinations with triplicates in each set.

* Not significantly different from the corresponding wild-type values.

** Statistically different at P<0.0001 when compared to the corresponding values obtained for wild type (WT).

Table 3

Effect of paromomycin (PR) on mitochondrial and cytoplasmic protein synthesis in promastigotes of wild-type and paromomycin-resistant *Leishmania donovani*

Treatment	Wild-type	PRr
Untreated control	100	100
PR (1 mM)	79 ± 3.5	98 ± 2.1
Cycloheximide (70 µM)	51 ± 3.5	38 ± 11.3
PR + Cycloheximide	29 ± 9.8	42 ± 12.7
Chloramphenicol (1 mM)	59 ± 5.7	66 ± 5.6
PR + Chloramphenicol	29 ± 1.4	66 ± 2.1

Cells were treated as described in materials and methods. Results are expressed as percentage incorporation of isoleucine in treated cells in comparison to the untreated controls. Data represents mean ± SD of duplicate values from two independent experiments.